

## Accepted Manuscript

Development of nanostructured lipid carriers containing salicyclic acid for dermal use based on the Quality by Design method

A. Kovács, Sz. Berkó, E. Csányi, I. Csóka



PII: S0928-0987(16)30560-7

DOI: doi: [10.1016/j.ejps.2016.12.020](https://doi.org/10.1016/j.ejps.2016.12.020)

Reference: PHASCI 3837

To appear in: *European Journal of Pharmaceutical Sciences*

Received date: 7 September 2016

Revised date: 24 November 2016

Accepted date: 15 December 2016

Please cite this article as: A. Kovács, Sz. Berkó, E. Csányi, I. Csóka , Development of nanostructured lipid carriers containing salicyclic acid for dermal use based on the Quality by Design method. The address for the corresponding author was captured as affiliation for all authors. Please check if appropriate. Phasci(2016), doi: [10.1016/j.ejps.2016.12.020](https://doi.org/10.1016/j.ejps.2016.12.020)

This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

## **Development of nanostructured lipid carriers containing salicylic acid for dermal use based on the Quality by Design method**

*A. Kovács\*, Sz. Berkó, E. Csányi, I. Csóka*

Institute of Pharmaceutical Technology and Regulatory Affairs, University of Szeged, Faculty of Pharmacy, Eötvös str. 6, Szeged H-6720, Hungary

\*Corresponding author:

Anita Kovács

Institute of Pharmaceutical Technology and Regulatory Affairs, University of Szeged,  
Eötvös str. 6, Szeged H-6720, Hungary, Tel.: +36 62 54 55 73,  
e-mail: anita.kovacs@pharm.u-szeged.hu

Szilvia Berkó

Institute of Pharmaceutical Technology and Regulatory Affairs, University of Szeged,  
Eötvös str. 6, Szeged H-6720, Hungary,  
e-mail: berko@pharm.u-szeged.hu

Erzsébet Csányi

Institute of Pharmaceutical Technology and Regulatory Affairs, University of Szeged,  
Eötvös str. 6, Szeged H-6720, Hungary,  
e-mail: csanyi@pharm.u-szeged.hu

Ildikó Csóka

Institute of Pharmaceutical Technology and Regulatory Affairs, University of Szeged,  
Eötvös str. 6, Szeged H-6720, Hungary,  
e-mail: csoka@pharm.u-szeged.hu

**Abstract:**

The aim of our present work was to evaluate the applicability of the Quality by Design (QbD) methodology in the development and optimization of nanostructured lipid carriers containing salicylic acid (NLC SA). Within the Quality by Design methodology, special emphasis is laid on the adaptation of the initial risk assessment step in order to properly identify the critical material attributes and critical process parameters in formulation development.

NLC SA products were formulated by the ultrasonication method using Compritol 888 ATO as solid lipid, Miglyol 812 as liquid lipid and Cremophor RH 60<sup>®</sup> as surfactant. LeanQbD Software and StatSoft. Inc. Statistica for Windows 11 were employed to identify the risks.

Three highly critical quality attributes (CQAs) for NLC SA were identified, namely particle size, particle size distribution and aggregation. Five attributes of medium influence were identified, including dissolution rate, dissolution efficiency, pH, lipid solubility of the active pharmaceutical ingredient (API) and entrapment efficiency.

Three critical material attributes (CMA) and critical process parameters (CPP) were identified: surfactant concentration, solid lipid/liquid lipid ratio and ultrasonication time. The CMAs and CPPs are considered as independent variables and the CQAs are defined as dependent variables. The 2<sup>3</sup> factorial design was used to evaluate the role of the independent and dependent variables. Based on our experiments, an optimal formulation can be obtained when the surfactant concentration is set to 5%, the solid lipid/liquid lipid ratio is 7:3 and ultrasonication time is 20 minutes. The optimal NLC SA showed narrow size distribution ( $0.857 \pm 0.014$ ) with a mean particle size of  $114 \pm 2.64$  nm. The NLC SA product showed a significantly higher *in vitro* drug release compared to the micro-particle reference preparation containing salicylic acid (MP SA).

**Keywords:** NLC, Quality by Design, risk assessment, critical quality attributes, 2<sup>3</sup> factorial design

**Abbreviations:**

API, Active Pharmaceutical Ingredient

CMAs, Critical Material Attributes

CCS, Critical Control Strategy

CPPs, Critical Process Parameters

CQAs, Critical Quality Attributes

DoE, Design of Experiments

DS, Design Space

ICH, International Council for Harmonisation

LD, Laser diffraction

MP-SA, Micro-sized lipid particle containing salicylic acid

Nanostructured lipid carriers containing salicyclic acid (NLC SA)

NSAID, Non-steroidal anti-inflammatory drug

PAT, Process Analytical Technology

QbD, Quality by Design

QRM, Quality Risk Management

QTPP, Quality Target Product Profile

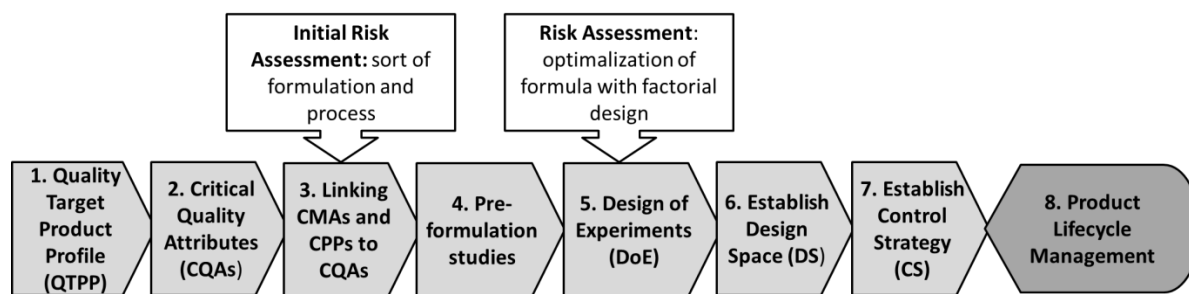
REM, Risk estimate matrix

SA, Salicyclic acid

## 1. Introduction

Application of the „quality by design” methodology according to the ICH Q8 guideline is a fairly new approach in the development process of new pharmaceutical products. The QbD approach is usefull in the daily pharmaceutical industrial practice (ICH Q8, 2009). It is a systematic approach that begins with predefined objectives, and emphasizes product and process understanding, as well as process control, based on sound science and quality risk management. The process starts with the determination of the quality target product profile (QTPP) and the critical quality attributes (CQAs). Critical material attributes (CMAs) and critical process parameters (CPPs) are identified, as well as risk assessment is carried out (ICH Q9, 2006) in order to identify the material attributes and process parameters which potentially affect product CQAs.

Therefore, the QbD approach is more proactive and refers to a systematic process compared to the mainly empirical methodologies used earlier (Beg, S. et al., 2015, Shah, B. et al., 2015, Xu, X. et al., 2011 Xu, X. et al., 2012, Kan, S. et al., 2014, Wang, J. et al, 2015, Kovacs, A et al., 2016). Risk assessment and the Design of Experiments (DoE) techniques within the risk assessment process are key elements of QbD methodology (Fig.1). Risk assessment includes the identification of potential hazards plus the analysis and evaluation of the risks associated with the exposure to these hazards (Beg, S. et al., 2015). The ICH Q9 guideline lists several quality management tools (e.g. Ishikawa diagram, Pareto analysis, risk estimate matrix etc.) and favors the Design of Experiments (DoE) techniques (e.g. screening techniques, interaction effect techniques etc.) (Singh et al 2011).



**Fig. 1.** Flow chart of Quality by Design approach in formulation development

Our hypothesis that the adaptation of the QbD based dosage form development in the early research phase leads to a more systematic R&D approach, and consequently gives a greater potential to the final product to reach the market earlier, has already been proved for newly developed nasal formulas (Pallagi, E. et al., 2015).

Now the QbD approach is applied to the development of a complex dosage form, namely for Solid Lipid Nanoparticles (SLN) and Nanostructured Lipid Carriers (NLCs), which are potent drug delivery systems for e.g. dermal use. These systems are derived from o/w emulsions by replacing the lipophilic liquid phase with solid lipid(s), which are dispersed in an external aqueous phase with suitable emulsifier(s) (Baroli, B., 2010). The active substance is present in a dissolved or dispersed form, and is characterized by a size range of 40 to 1000 nm (Müller, R.H. et al., 2002, Pardeike, J., et al., 2009, Subedi, R.K., et al., 2009). Nanostructured Lipid Carriers (NLCs) is the term used for second generation solid lipid nanoparticles that contain a lipid matrix of mixed solid and liquid lipids.

The main advantages of these systems include the following: (1) they are ideal carriers to incorporate low water-soluble active substances and to stabilize oxidation-/photo-sensitive materials (Müller, R.H. et al., 2002, Pardeike, J., et al., 2009); (2) as dermally applied systems (McGrath, J.A. and J. Uitto, J., 2010, Prow, T.W., et al., 2011, Cevc, G. and Vierl, U., 2010) they ensure close contact with the lipid bilayer of the stratum corneum, resulting in a more efficient and deeper drug penetration into the skin layers (Yang, X., et al., 2013). Their occlusive properties resulting from film formation were also reported for NLC formulations (Wissing, S.A. and Müller, R.H., 2002a, Wissing, S.A. and Müller, R.H., 2002b), as well as their protective capacity against environmental effects such as UV radiation (also called physical UV filter function) (Müller, R.H. et al., 2014, Lacatusu, I., et al., 2011).

Salicylic acid (SA) as an NSAID drug with antifungal, anti-infective and keratolytic properties was used as model drug because of its wide therapeutic use (e.g. for the treatment of acne, psoriasis, callouses, corns, keratosis pilaris and warts), its physicochemical properties (molecular weight <400 Da, log P = 2–3.8), and also because SA is poorly water soluble,

thereby it is a good candidate for our studies. Incorporating salicylic acid into NLC nanoparticles may protect against the irritating side effects and may enhance skin penetration, thereby it is possible to achieve the same effect with less amount of active substance compared to conventional pharmaceutical dosage forms (Casanova, F., 2015).

The first aim of our present work was to adapt the QbD approach in the optimization and development of stable salicylic acid loaded nanostructured lipid carriers for dermal use. As the first step of this process, the QTPP and CQAs were determined, then an initial risk assessment was carried out to optimize the material attributes (CMAs) and process parameters (CPPs) affecting the critical quality attributes (CQAs) of SA-containing NLC systems. Our further aim was the practical implementation of the  $2^3$  factorial design method as a risk assessment tool in order to determine the optimal composition for the formulation. Thirdly, methods of monitoring selected critical parameters as “in-process” and as final product quality control measures were also adopted and recommended for the NLCs.

## **2. Materials and Methods**

### **2.1. Materials**

Salicylic acid was purchased from Sigma-Aldrich (USA), Compritol 888 ATO (glyceryl behenate/dibehenate) was supplied by Azelis Hungary Ltd. (Hungary), Miglyol 812 (caprylic/capric triglyceride) was provided by Sasol GmbH (Germany) and Cremophor RH 60 (PEG-60 hydrogenated castor oil; HLB value:15-17) was kindly supplied by BASF SE Chemtrade GmbH (Germany). Bidistilled water was used throughout the experimental work. All other chemicals were of analytical grade unless otherwise stated.

### **2.2 Methods**

#### **2.2.1. Definition of the QTPP**

The initial step of the QbD based development is to define the target product profile (TPP) and the selected QTPP based on requirements of stakeholders (patient expectations, industrial and regulatory aspects). TPP includes the definition of the route of administration, the dosage form, maximum and minimum doses, appearance etc. QTPPs are quality, safety and efficiency features of a product, such as stability, drug release profile, pharmacokinetic attributes, purity, bioavailability etc. depending on the specific dosage form, the route of administration and the therapeutic aim (ICH Q8, 2009).

#### **2.2.2. Determination of the CQAs**

The second step of the QbD based development is to define and summarize the quality attributes of a product which have to be ensured during development and production in order to achieve the required final quality. The CQAs are derived from the QTPP and are based on prior product knowledge. CQAs include the physical, chemical, biological, or microbiological properties that should be within an appropriate limit, range, or distribution to ensure the desired product quality (ICH Q8, 2009). CQAs are dependent on the raw materials on the in-process materials used, as well as on the final drug product. The CQAs may include particle/droplet size, drug release, entrapment efficiency, purity, pH, viscosity etc. Regarding that the size range of the NLC system is between 10–1000 nm (“nano” range), particle size and particle size distribution are further risk parameters in the present case.

### 2.2.3. Determination of the CMAs and CPPs

The third step of the QbD based development is to determine the material attributes and process parameters (based on prior knowledge, literature data and previous laboratory experiments) that may influence product CQAs, and also to find the functional relationships between these material attributes and process parameters related to product CQAs (ICH Q8, 2009). Factors of interest include properties of the API, properties of the excipients (e.g., lipid and surfactant), and process parameters (e.g. homogenization time and temperature).

### 2.2.4. Initial risk assessment: screening design

Risk assessment is the focus of the QbD based product development. Risk assessment consists of identification, analysis and evaluation of risks detected for a given formulation (ICH Q9, 2006). In case of a dermally used NLC system, the following failure modes/risk factors may occur: inadequate particle size of the drug product (NLC preparation), inadequate drug substance solubility, inadequate *in vitro* drug release rate or time, inhomogeneity of the drug product, kinetic instability (e.g. aggregation, phase separation), and incompatibility between the drug substance and the excipients (Chang et al., 2013, EMA/CHMP, 2013). These risks are best evaluated by using an Ishikawa diagram. The risk estimate matrix allows the evaluation of the critical factors from a large number of parameters affecting the CQAs, allowing to reduce the number of risk factors to be studied in the experimental phase. The Pareto chart shows the relationships between CMAs or CPPs and CQAs, respectively and it can be used to determine the most critical parameters to be controlled for during the drug development process. Screening of parameters and defining the critical control points were carried out by the LeanQbD™ software (QbD Works LLC, Fremont, CA, USA) (Kovacs, A. et al., 2016, Pallagi, E. et al., 2015).

### 2.2.5. Preparation of the NLC formulation

NLC formulations were prepared by ultrasonication method using an UP 200s Ultrasonic Processor (Hielscher Ultrasonics GmbH, Germany). Salicylic acid equaling to 4 w/w% was dissolved in the melted blend of solid lipid (Compritol ATO 888) and liquid lipid (Myglyol 812) at 75 °C. Salicylic acid concentration was 0.4 w/w% in final formulations and total lipid concentration was 10 w/w% in each samples. The surfactant was dissolved in bidistilled water at the same temperature. The aqueous phase was added to the lipid phase and was stirred with Ultra Turrax T25 (IKA-Werke, Germany) for 60 seconds at 10,000 rpm. The pre-emulsion was subjected to ultrasonication at a continuous mode at 70% amplitude for 10 or 20 minutes. Blank NLC was prepared with the same procedure, but without adding the active agent. The reference micro-sized particle preparation (MP SA) was prepared using the same composition and the same procedure, but no ultrasonication.

### 2.2.6. Preformulation study

*Analysis of the API in the NLC compositions with X-ray powder diffraction (XRPD)*

The XRPD analysis was performed with a Bruker D8 Advance diffractometer system (Bruker AXS GmbH, Karlsruhe, Germany) with Cu K  $\alpha$  radiation ( $\lambda = 1.5406 \text{ \AA}$ ). The samples were scanned at 40 kV and 40 mA from 3 to 40  $2\theta$ , at a scanning speed of 0.1/s and a step size of 0.010. Before the measurement, lipid mixtures and lipid-drug mixtures were melted and left to cool down to room temperature. The solid lipid was examined as received, without any treatment.

### 2.2.7. Design of experiments using the $2^3$ factorial design

In a factorial experiment (factorial design, FD) all levels (x) of a given factor (k) are combined with all levels of every other factor included in the experiment, and the total number of experiments are  $x^k$  (Singh et al., 2011). In this case the  $2^3$  full factorial design was used to determine the optimal ingredient ratio and preparation settings for the SA loaded NLCs. The effects of the factors are examined at two levels (+1 and -1). The levels are chosen based on literature data and previous laboratory experiments (Table 1A). For the experimental design process, eight different NLC samples (Table 1B) were prepared in triplicates according to the  $2^3$  full factorial design methodology. Statistical data analysis was performed using StatSoft. Inc. Statistica for Windows 11.



**Table 1.** (A) Values of the examined independent variables (X1, X2 and X3) and types of dependent variables (Y1, Y2). (B) Summarizes the compounds of the prepared formulations.

**A**

Type of variables	Levels	
Independent variables	Low (-1)	High (+1)
X1: surfactant concentration (w/w%)	1	5
X2: solid/liquid lipid ration	7:3	9:1
X3: ultrasonication time (min)	10	20
<b>Dependent variables</b>		
Y1: particle size		
Y2: particle size distribution		

**B**

	NLC 1	NLC 2	NLC 3	NLC 4	NLC 5	NLC 6	NLC 7	NLC 8
Cremophor RH 60 (w/w%)	1	1	5	5	1	1	5	5
Lipid ratio	9:1	7:3	9:1	7:3	9:1	7:3	9:1	7:3
Ultrasonication time (min)	10	10	10	10	20	20	20	20

## 2.2.8. Characterization of NLC SA

### Particle size analyzis

Determination of particles size and particle size distribution (span) were performed by laser diffraction (LD) using a Mastersizer 2000 (Malvern Instruments, UK). Three values, namely  $d(0.1)$ ,  $d(0.5)$ , and  $d(0.9)$  were evaluated, indicating that 10%, 50%, and 90% of the analyzed particles are below a certain size (volume distribution). The span value describing the width of the particle size distribution curve ( $((d(0.9)-d(0.1))/d(0.5))$ ) was also calculated. The measurement medium was purified water, with a refractive index of 1.33. The refractive index was set to 1.456.

### *In vitro* drug diffusion

The *in vitro* drug diffusion analysis was performed by using the dialysis bag method (Araújo, J., et al., 2012, Kheradmandnia, S., et al., 2010). 200  $\mu$ l of the NLC formulation was sealed in a Spectra/Por<sup>®</sup> 4 dialysis membrane with Spectra/Por<sup>®</sup> Closures (Spectrum Laboratories Inc., Rancho Dominguez, CA, USA), and placed into 25 ml of phosphate-buffered saline (PBS)

(pH = 7.4). The system's temperature was 32°C, and continuous stirring at 450 rpm was applied. After 0.5, 1, 2, 3, 4, 5 and 6 hours 1 ml samples of the bulk solution were taken. The withdrawn samples were replaced by 1 ml of PBS to maintain sink conditions. The samples were analyzed at 295 nm with a Unicam Evolution 201 UV/Vis spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA, USA). The reference preparation (MP SA) was subjected to the same procedure.

### 2.2.9. Statistical analysis

Results of the factorial design experiment were analyzed by the statistical software of Statistica for Windows software version 11. Statistical analysis of the *in vitro* data were carried out using the two-way analysis of variance test (Bonferoni's posttest comparison) by the Prim for Windows 5 Software (GraphPad Software Inc. La Jolla, CA, USA).

## 3. Results and discussion

### 3.1. Definition of QTPP and CQAs for NLC SA for dermal use

The first step of a QbD-based drug development process is defining the target product profile (TPP) and the selected QTPP. Using the QbD methodology, we aimed to develop and optimize a stable NLC SA formula that has a reduced SA concentration but a non-inferior or an enhanced therapeutic effect during dermal use. QTPP for the NLC system include therapeutic effectiveness, stability and the dissolution profile of sustained drug release. Therapeutic effectiveness of an NLC SA for dermal use depends on the bioavailability and the solubility of API. Solubility of the API is one of the most critical parameters affecting whether the desired drug concentration within the stratum corneum is achieved. Stability is another important parameter to ensure the safety and efficacy of the drug product. Particle size and particle size distribution are expected to influence stability. The mean particle size (d<sub>0.5</sub>) and particle size distribution width (span) together are suitable parameters to check the stability of the nanoparticles. The aggregation of the particles are clearly indicated by these increasing values during the preparation process and as well as the long term stability. Furthermore, particle size and solubility of the API in the lipid matrix are expected to influence drug release, and consequently skin transport as well (Sütő et al., 2015b). Therefore, particle size and solubility of the API are identified as CQAs. Additionally, numerous interactions may appear between the parameters. Risk assessment can help to identify these interactions and their effects can be evaluated. Table 2 lists the QTPP parameters with their targets and their justification.

**Table 2.** Quality Target Product Profile (QTPP) of SA containing NLC.

QTPP parameters	Target	Justification
Dosage form	NLC	Salicylic acid is poorly water soluble and has skin irritative properties. Nanostructured lipid carriers (NLC) overcome these problems. The small size of the NLCs ensures close contact between the lipid particles and the lipid bilayer of the stratum corneum, resulting in an increased penetration of the drug into the deeper layers of the skin.
Route of administration	Dermal	The advantage of dermal preparations is their local administration at the affected site, thereby avoiding systemic side effects appearing in case of oral administration.
Therapeutic effect	Anti-inflammatory	Salicylic acid is an NSAID with antifungal, anti-infective, keratolytic and anti-inflammatory properties. Its indications include e.g. hyperkeratotic skin disorders, acne, warts and calluses scalp conditions and fungal nail infections.
Stability (physical, chemical, biological)	No visible signs of aggregation at the time of preparation and within 1 months afterwards (at room temperature)	Stability is required in order to retain the therapeutic effect of the drug during shelf-life and is a crucial requirement for marketing authorization.
Dissolution profile	Sustained drug release	Sustained drug release can reduce the irritation caused by SA.
Dosage strength	0.4g/100g	Usual adult dosage of SA in topical preparations for acne is 0.5 to 2.0w/w% (FDA, 2011). From the lipid nanoparticle film applied to the skin, diffusion of drug molecules into skin layers is driven by the concentration gradient. Dermal penetration and therefore intradermal drug concentration is enhanced by the occlusive effect of the topical film. As a result of that, a lower dosage strength can produce the required therapeutic effect.
Container closure system	Appropriate for the dosage form	It is needed to ensure target shelf-life and the NLC's safety and is a requirement for marketing authorization.

After determining the QTPP parameters, the next step is identifying the quality attributes that have to be assured for the proper quality of the NLC SA product. The CQAs are derived from the QTPPs. The quality attributes identified include particle size, particle size distribution, aggregation, physical attributes of the drug product, dissolution rate (dissolution speed), dissolution efficiency (dissolution performance of the drug product), pH, solubility of the API, entrapment efficiency and viscosity. Table 3 illustrates the potential CQAs affecting the quality of the NLC SA formulations along with justification for each of them.

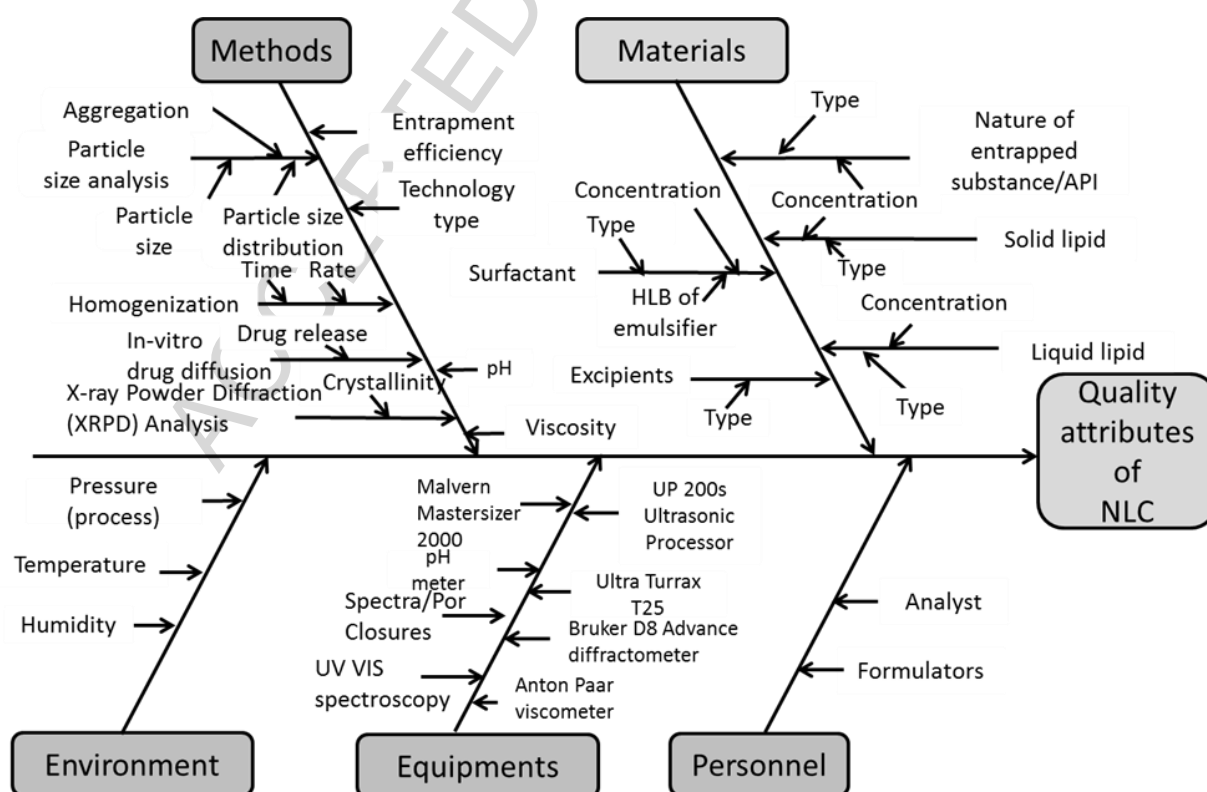
**Table 3.** Quality Attributes and CQAs of SA-containing NLC

Quality attributes	Target	Is it a CQA?	Justification
Physical attributes (colour, odour, appearance)	Opalescent to white, odourless dispersion	No	Physical attributes are not critical, because they are not directly linked to efficiency and patient safety.
Particle size	Mean particle size range: 100–200 nm	Yes	Smaller particle size allows easier penetration through the stratum corneum. It is considered as critical for the formula.
Particle size distribution	Span value $\leq 1$ (the span value refers to the width of the particle size distribution.)	Yes	Low span values indicate the narrow particle size distribution. Based on the work of the research group (Sütő, B. et al., 2015a ) we defined as the target, the span value should be $\leq 1$ . It is important for the stability of the NLC.
Dissolution rate	Sustained	Yes	Required for a long-lasting dermal effect.
Dissolution efficiency in 60 min	$\geq 50$ % and higher than MP	Yes	Dissolution performance is an indicator of drug release from the salicylic acid loaded NLC system. It is important for enhancing the therapeutic effect. A higher dissolution efficiency of the API results in enhanced therapeutic benefits.
Aggregation	d(0,9) range: 100 nm (0,1 $\mu$ m) to 200 nm (0,2 $\mu$ m)	Yes	The d(0,9) range shows during the cooling phase of production the particles preserved the separateness.. It is important for the stability of the NLC during the preparation process.

Solubility of the API in lipid matrix	High (approx. 90%)	Yes	It is an indicator of the dissolution efficacy of salicylic acid in lipid matrix. It is important for enhancing therapeutical effectiveness (Patel, J.N. et al., 2012).
Entrapment efficiency	min. 95%	Yes	It is an indicator of higher drug loading (Sütő et al. 2015b).
Viscosity of NLC dispersion	Range: 100–500 mPas	Yes	Viscosity may affect drug release and stability of the NLC.
pH	pH value: 4-8	Yes	pH can influence the loading during the preparation.

### 3.2. Initial Risk Assessment

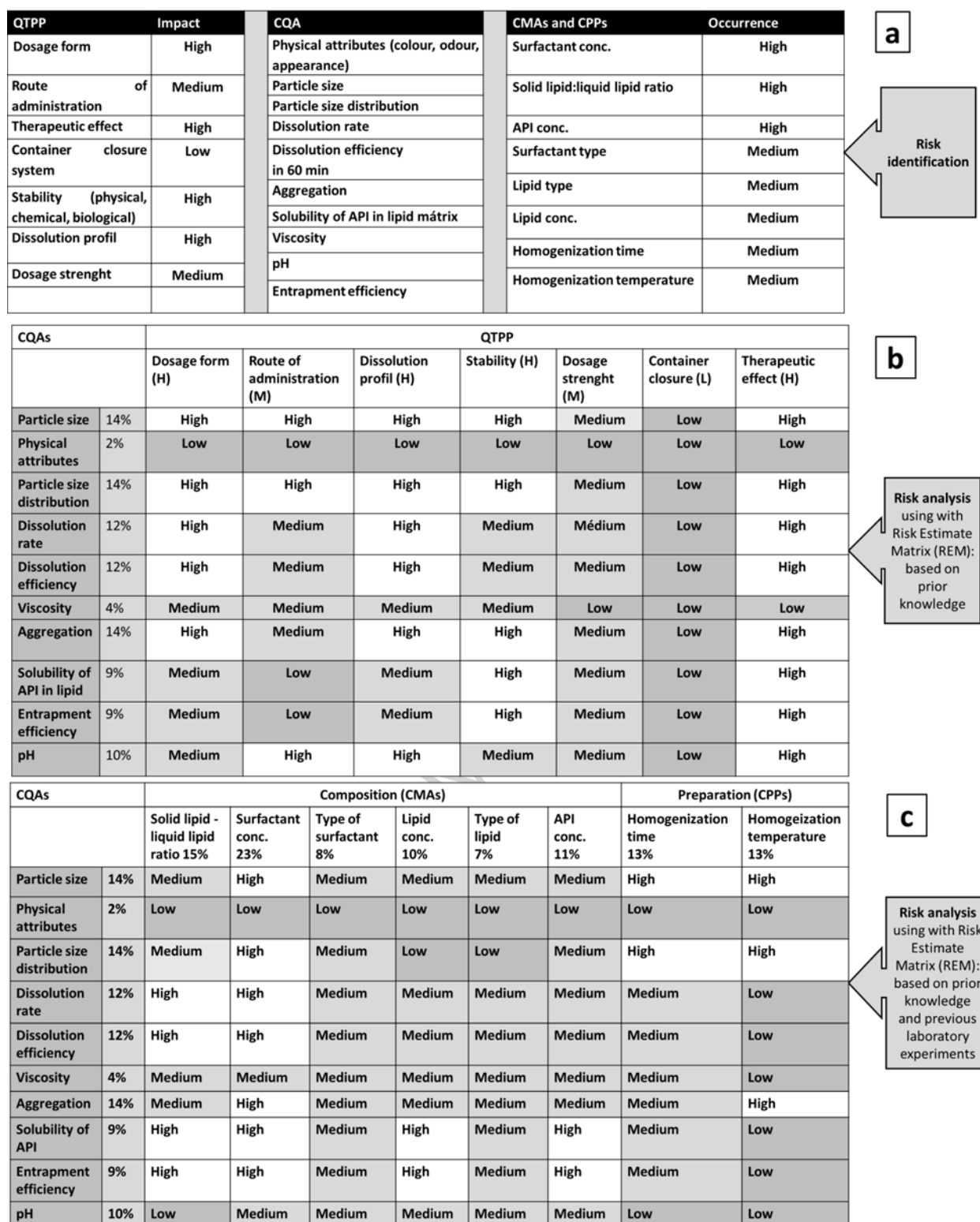
Risk assessment refers to the quantitative or qualitative estimate of the risks related to the NLC containing salicylic acid. Risk factors are ranked by risk analysis. An Ishikawa (fishbone) diagram was constructed to identify the effects of key material attributes and process parameters for the development of NLC containing salicylic acid (Fig. 2.). It illustrates the causes and sub-causes affecting the CQAs of the NLC system.



**Fig. 2.** Ishikawa diagram: cause-and-effect relationship between the material and process variables for the quality attributes of NLC SA

### **Risk estimate matrix**

After identifying the QTPPs and the CQAs, the following step is determining the critical material (drug substance, excipients) and process parameters. The selected parameters are shown in Fig.3A, while Fig.3B presents the risk estimate matrix (REM) of interdependence rating between the CQAs and QTPPs for NLC SA, by assigning low, medium and high values to each of them. The risk estimation matrix (REM) represents the potential risk(s) associated with each material attribute(s) and/or process parameter(s) that have a potential effect on the CQAs. For the probability rating a 1(low)-3(medium)-9(high) scale was used, considering all QTPP, CMA and CPP factors and their relationships to the CQAs. The following factors were found to influence product quality: particle size (14%), particle size distribution (14%), particle aggregation (14%), the drug's dissolution profile (rate: 12%; efficiency: 12%), solubility of the API (9%), pH (10%) and entrapment efficiency (9%). Fig.3C illustrates the risk associated with CMAs and CPPs. Eight CMA and CPP parameters were screened to determine their influence on the CQAs during the preparation of NLC. Probability rating was done the same way as it was for the previous REM. The interdependence between the QTPPs and CQAs, just as between the CQAs and CMAs and CPPs, was structured, and evaluated one by one with a LeanQbD Software.

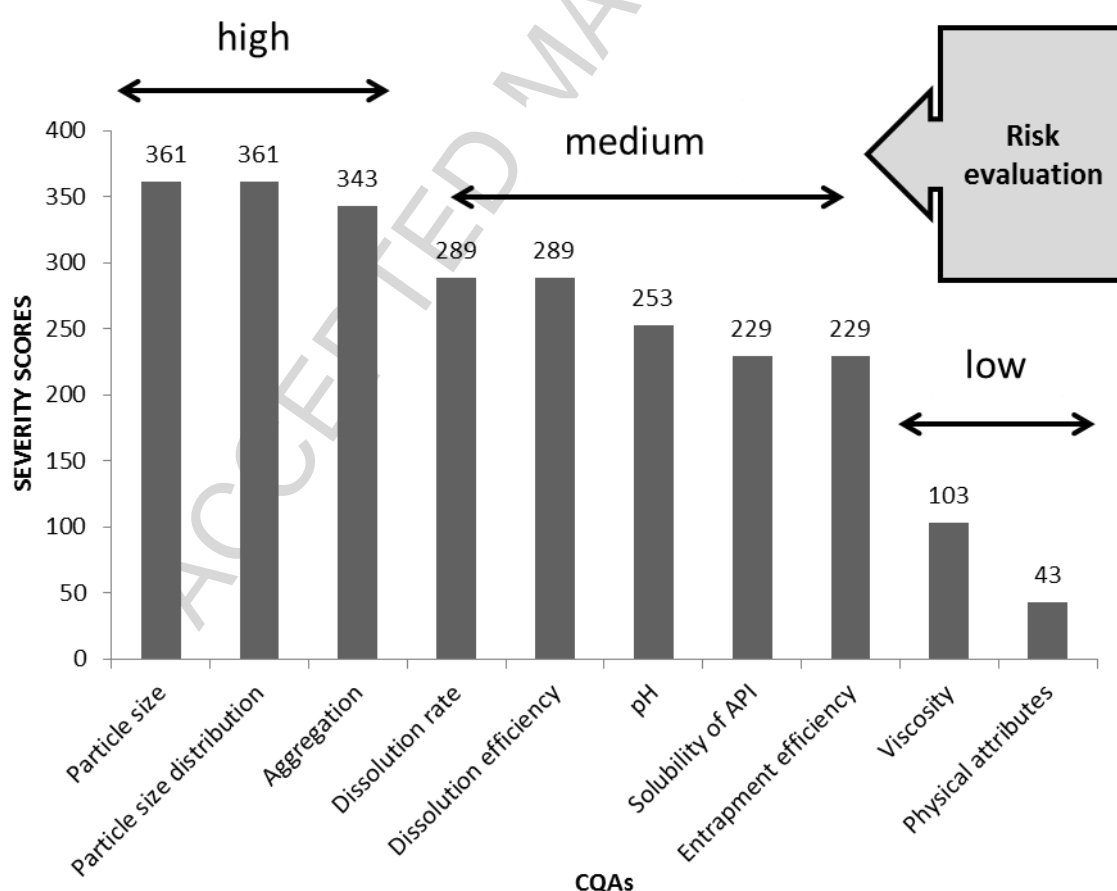


**Fig. 3.** Selected QTPPs, CMAs, CPPs and CQAs and their interdependence rating with risk estimation matrix (Lean-QbD Software): Low=low risk parameter; Medium=medium risk parameter; High= high risk parameter

Based on the REM results, a Pareto chart (Fig. 4.) was generated showing the severity scores of CQAs that lead us to the following conclusions: particle size, partical size distribution and

aggregation were the CQAs with the highest severity score (>300) suggesting that these are the most critical parameters influencing the quality of NLC SA. The next highest category of severity scores is 200–299, and include the following parameters: dissolution rate, dissolution efficiency, pH, solubility of the API and entrapment efficiency, suggesting that these parameters have medium influence on the final product's quality. Viscosity and physical attributes (colour, odour, appearance) were characterized by severity scores below 200, thereby having a low impact on the quality of the drug product. In this study the severity score threshold was set to 200, and any risk factors with a severity score above 200 was considered as a potential risk factor that should be paid attention during the development of the NLC. Based on the results of the risk assessment the particle size, the particle size distribution and the aggregation were the high risk parameters, therefore these parameters have been investigated in the first step which are only examined in this paper.

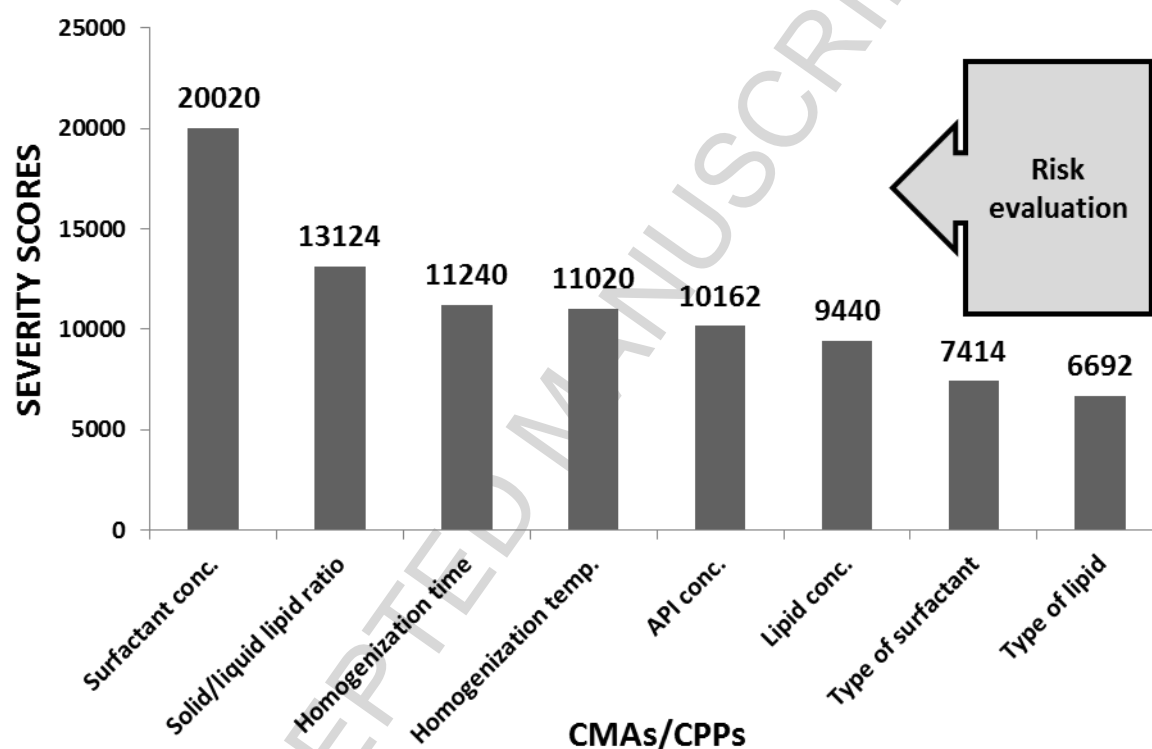
The most critical parameters will be the dependent variables of the  $2^3$  factorial design experiment which is the next phase of the QbD-based drug development process.



**Fig. 4.** Pareto analysis of the identified CQAs



The Pareto chart in Fig. 5 shows the relationships between the CMAs, the CPPs and the CQAs, respectively, illustrating the most critical parameters which have to be paid attention during the drug development process. Based on the results of the initial risk assessment, three factors, namely surfactant concentration, the solid lipid/liquid lipid ratio and ultrasonication time were found to be highly critical factors for CQAs and for QTPP. These factors were further analysed as independent variables with the  $2^3$  factorial design in order to further optimize drug formulation. This way both time and costs can be saved by omitting unnecessary experiments.

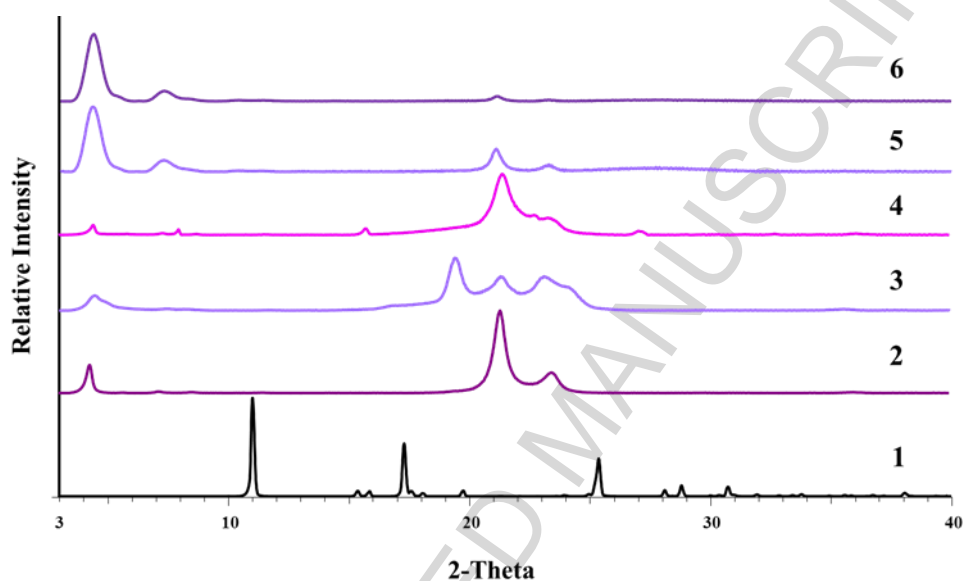


*Fig. 5. Pareto analysis of selected material and process parameters*

### 3.3. Preformulation studies: X-ray powder diffraction (XRPD) analysis of the components

XRPD measurements were carried out to determine the crystal structure of salicylic acid in the solid lipid, in the solid lipid/liquid lipid mixture and in the NLC. Diffractograms of (1) the pure API, (2) Compritol 888 ATO as solid lipid, (3) the solid lipid/liquid lipid mixture, (4) the physical mixture of components of NLC, (5) blank NLC and (6) NLC SA are shown in Fig. 6.

The diffractogram of salicylic acid (1) confirms that the drug is in the crystalline state. Comparing the diffractograms of the pure solid lipid (2) and the lipid mixture (3), it is clearly visible that the crystallinity of Compritol 888 ATO decreases and its structure becomes less ordered after the addition of Miglyol 812. This predicts a good drug loading ability of the lipid matrix. On the diffractogram of the physical mixture (4) no peaks of the drug are visible, which means that salicylic acid is dissolved in the lipid mixture. Diffractograms of the blank- and drug-loaded NLC formulations (5, 6) show a decreased crystallinity of the lipid matrix. Peaks of the drug cannot be seen on the diffractogram of NLC SA, which indicates the dissolved state of the active agent in the final formulation.



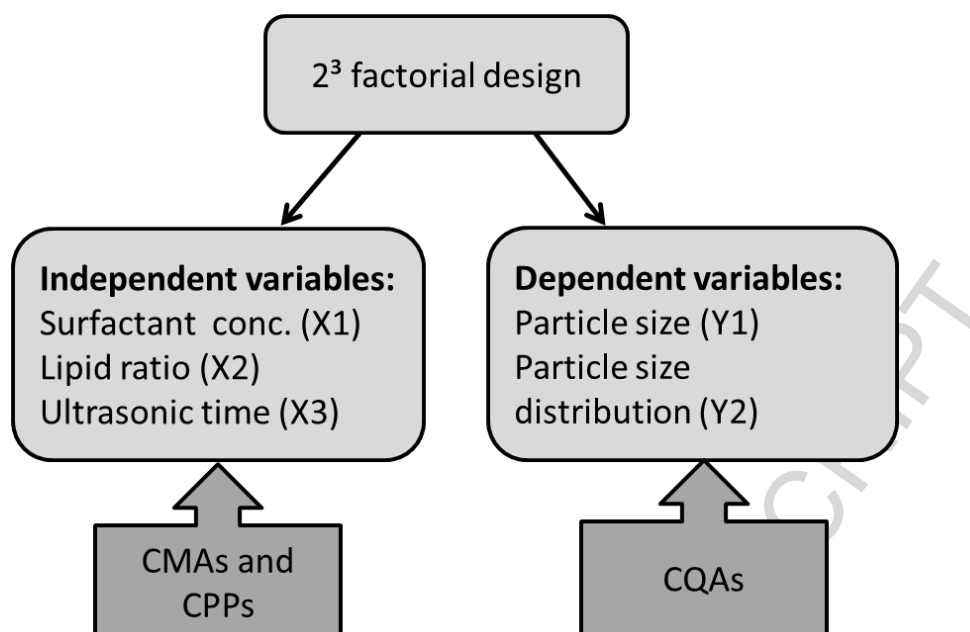
**Fig. 6.** Diffractograms of pure API (1), Compritol 888 ATO (2), lipid mixture of 7:3 ratio (3), physical mixture (4), blank NLC (5) and NLC SA (6).

### 3.4. Experimental design

#### 3.4.1 The 2<sup>3</sup> factorial design

DoE (Design of Experiments) is a risk assessment tool to detect the possible interactions between the factors affecting the drug development process and thus the quality of the final product. It is an effective method for an objective interpretation and the implementation of the results, considering simultaneous parameter changes. Three independent variables and two dependent variables were selected based on the initial risk assessment. The effect of the independent variables (X1 – surfactant concentration; X2 – solid lipid/liquid lipid ratio, and X3 – ultrasonication time) on quality attributes of NLC dependent variables (Y1 – mean

particle size (d0,5), and Y2 – particle size distribution (span) were investigated using the 2<sup>3</sup> factorial design (Fig. 7.).



**Fig. 7.** Selected parameters for the 2<sup>3</sup> factorial design based on the initial risk assessment

### 3.4.2. Particle size analysis

Particle size and particle size distribution of the prepared NLC system were measured by laser diffraction (LD). The mean particle size d(0.5) of the NLC systems varied from 0.116  $\mu\text{m}$  to 21.574. The span values varied from 0.874 to 5.646 (Table 4).

**Table 4.** Values of the independent (X1, X2 and X3) and dependent factors (Y1 and Y2) examined

Sample name	Surfactant concentration % (w/w)	Solid lipid/liquid lipid ratio	Ultrasonication time (min)	Particle size ( $\mu\text{m}$ )	Particle size distribution (span)
NLC 1	1(−1)	9:1(+1)	10(−1)	12.735	3.023
NLC 2	1(−1)	7:3(−1)	10(−1)	7.669	5.279
NLC 3	5(+1)	9:1(+1)	10(−1)	0.121	1.074
NLC 4	5(+1)	7:3(−1)	10(−1)	0.118	1.034
NLC 5	1(−1)	9:1(+1)	20(+1)	21.574	3.032
NLC 6	1(−1)	7:3(−1)	20(+1)	14.954	5.646
NLC 7	5(+1)	9:1(+1)	20(+1)	0.121	1.025

NLC 8	5(+1)	7:3(-1)	20(+1)	0.116	0.874
-------	-------	---------	--------	-------	-------

Salicylic acid concentration was 0.4 w/w% and total lipid concentration was 10 w/w% in each samples. The nanometer range could be reached in formulations NLC 3, 4, 7 and 8. Span values of these four samples show a narrow particle size width (low span values indicate the narrow particle size distribution). Samples NLC1, 2, 5 and 6 aggregated or became semisolid shortly after production, and their particle size was in the micrometer range. For these samples, span values indicate a broad, polydisperse particle size distribution.

### 3.4.3. Statistical analysis of data

Based on the 8 samples, the graphical results of the statistical analysis for mean particle size ( $d(0.5)$ ) and particle size distribution are shown in Fig. 8-9. The probability values (p-value) calculated by regression analysis are shown in Table 5.

**Table 5.** Results of the statistical analysis for mean particle size ( $d(0.5)$ )

Factor	Effect	t(1)	p	Coefficient	Standard Error Coefficient
(1) $X_1$	-14.1140	-36.3763	0.017497	-7.05700	0.194000
Mean/Intercept	7.1760	36.9897	0.017207	7.17600	0.194000
1 by 3	-4.0315	-10.3905	0.061081	-2.01575	0.194000
(3) $X_3$	4.0305	10.3879	0.061097	2.01525	0.194000
(2) $X_2$	2.9235	7.5348	0.084000	1.46175	0.194000
1 by 2	-2.9195	-7.5245	0.084114	-1.45975	0.194000
2 by 3	0.3890	1.0026	0.499181	0.19450	0.194000

It can be seen factor  $X_1$  (surfactant concentration) on its own, as well as the three examined independent factors combined (surfactant concentration ( $X_1$ ), solid lipid/liquid lipid ratio ( $X_2$ ) and sonification time ( $X_3$ )) exert a significant effect ( $p < 0.05$ ) on the mean particle size ( $d(0.5)$ ). The coupled factors were also tested, but did not give a significant effect (Table 5).

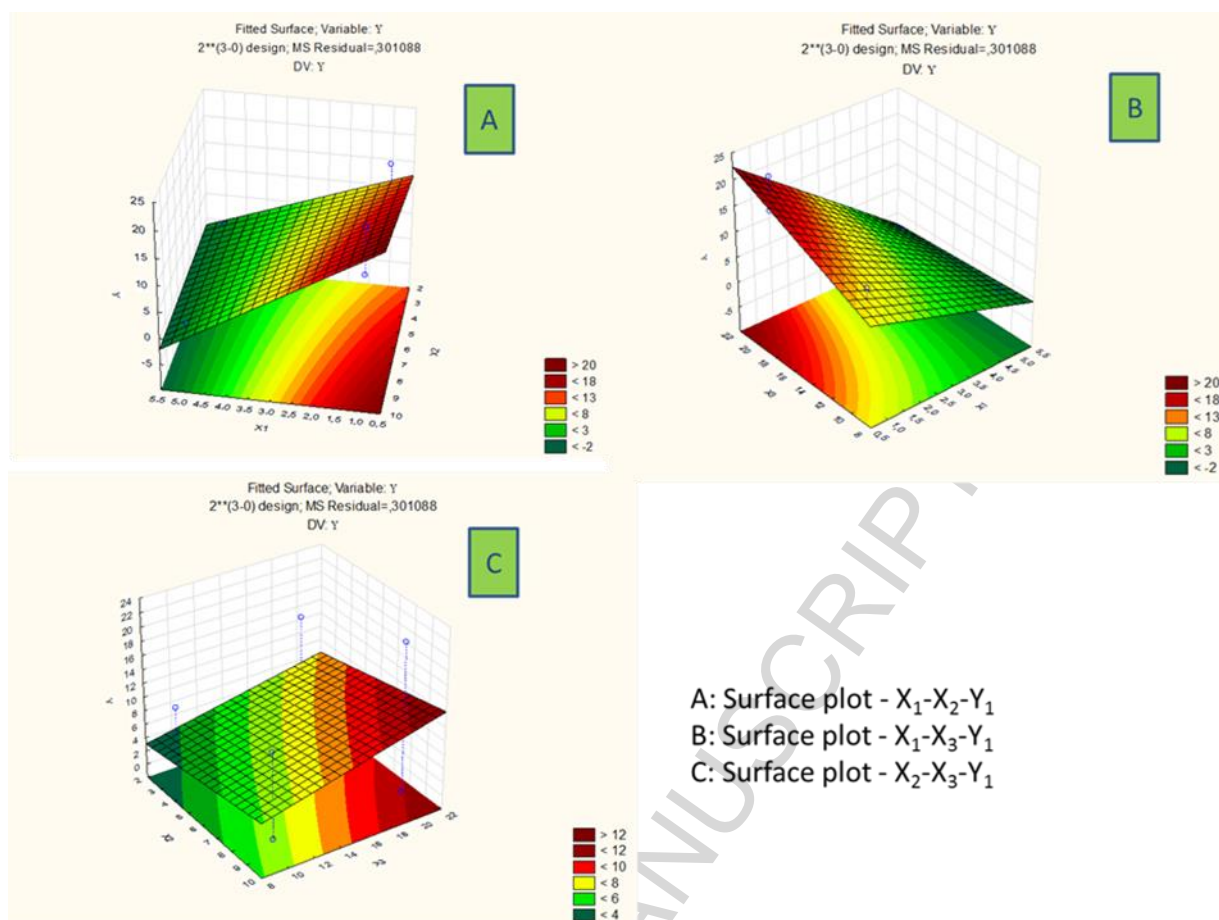
The mathematical model is shown in the following equation (Eq (1)) with good correlation  $R^2 = 0.9994$ .

$$Y_1 = 7.17600 - 7.05700 \cdot X_1 + 1.46175 X_2 + 2.01525 X_3 - 1.45975 X_{12} - 2.01575 X_{13} + 0.19450 X_{23} \quad \text{Eq (1)}$$

A positive sign indicates a synergistic effect on the dependent factor examined, whilst a negative sign represents an antagonistic effect. Factor X1 (surfactant concentration) was indirectly proportional to the mean particle size (d(0.5)). The influence of X2 and X3 factors on factor Y1 were not significant, while the negative sign of effect in case of factor X2 indicates an indirectly proportional and the positive sign of effect in case of X3 predicts a directly proportional relationship. These results are in accordance with the response surface plot (Fig. 8-9) and the Pareto chart (Fig. 10).

The slope of the diagram in Fig. 8A indicates that the smallest particle size is achieved at a surfactant concentration of 5 w/w%, while there is no difference between the 9:1 and 7:3 lipid ratios. Surfactant concentration is also significant in Fig. 8B, illustrating that the smallest particle sizes results from the highest surfactant concentration (5w/w%) and a higher ultrasonication time (20 min). Based on Fig. 8C a lower lipid ratio (7:3) and a shorter ultrasonication time (10 min) produces the smallest particle size

The statistical analysis of the independent factors for particle size distribution (span) shows similar results for mean particle size (d(0.5)) as shown in Table 6 and Fig. 9-10.



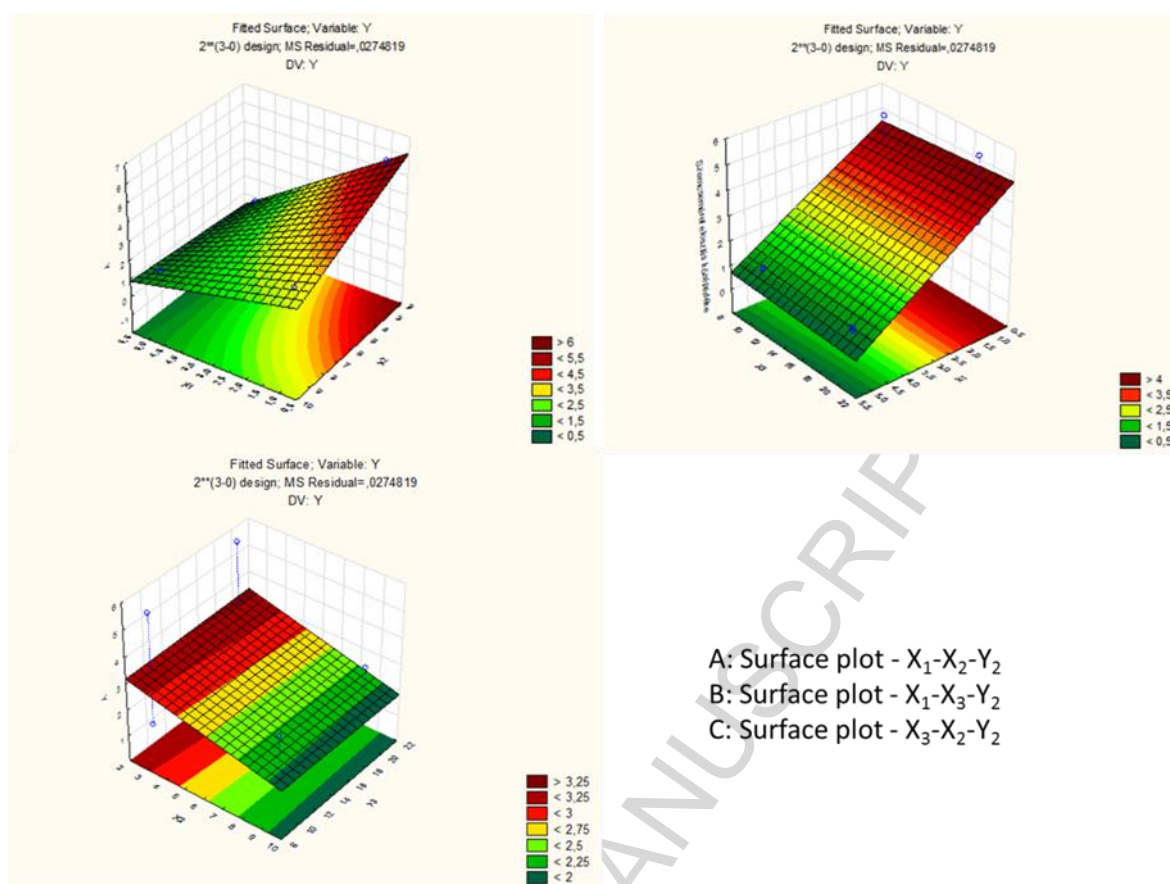
**Fig.8.** Response surface (3D) plot of the effects of variables on mean partical size of prepared NLC SA.

**Table 6.** Statistical analysis of particle size distribution (span).

Factor	Effect	t(1)	p	Coefficient	Standard Error Coefficient
<b>X1</b>	<b>-3.24318</b>	<b>-27.6670</b>	<b>0.023000</b>	<b>-1.62159</b>	<b>0.058611</b>
<b>Mean/Intercept</b>	<b>2.62327</b>	<b>44.7573</b>	<b>0.014221</b>	<b>2.62327</b>	<b>0.058611</b>
1 by 2	1.26546	10.7955	0.058803	0.63273	0.058611
X2	-1.16957	-9.9775	0.063593	-0.58479	0.058611
1 by 3	-0.14675	-1.2519	0.429076	-0.07338	0.058611
2 by 3	-0.06181	-0.5273	0.691071	-0.03091	0.058611
X3	0.04183	0.3568	0.781811	0.02091	0.058611

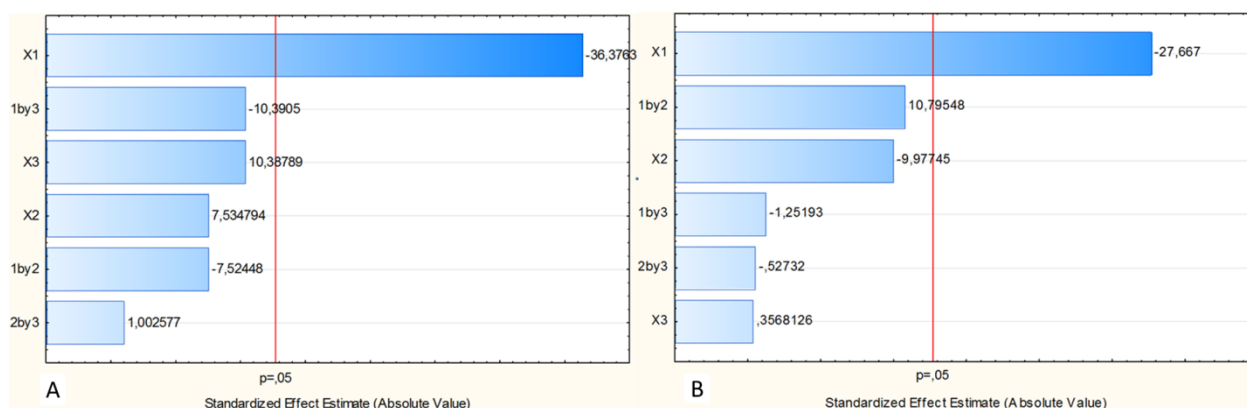
The mathematical model is shown in the following equation (Eq (2)) with good correlation  $R^2 = 0.99898$ .

$$Y_2 = 2.62327 - 1.62159 X_1 - 0.58479 X_2 + 0.02091 X_3 + 0.63273 X_{12} - 0.07338 X_{13} - 0.03091 X_{23} \quad \text{Eq (2)}$$



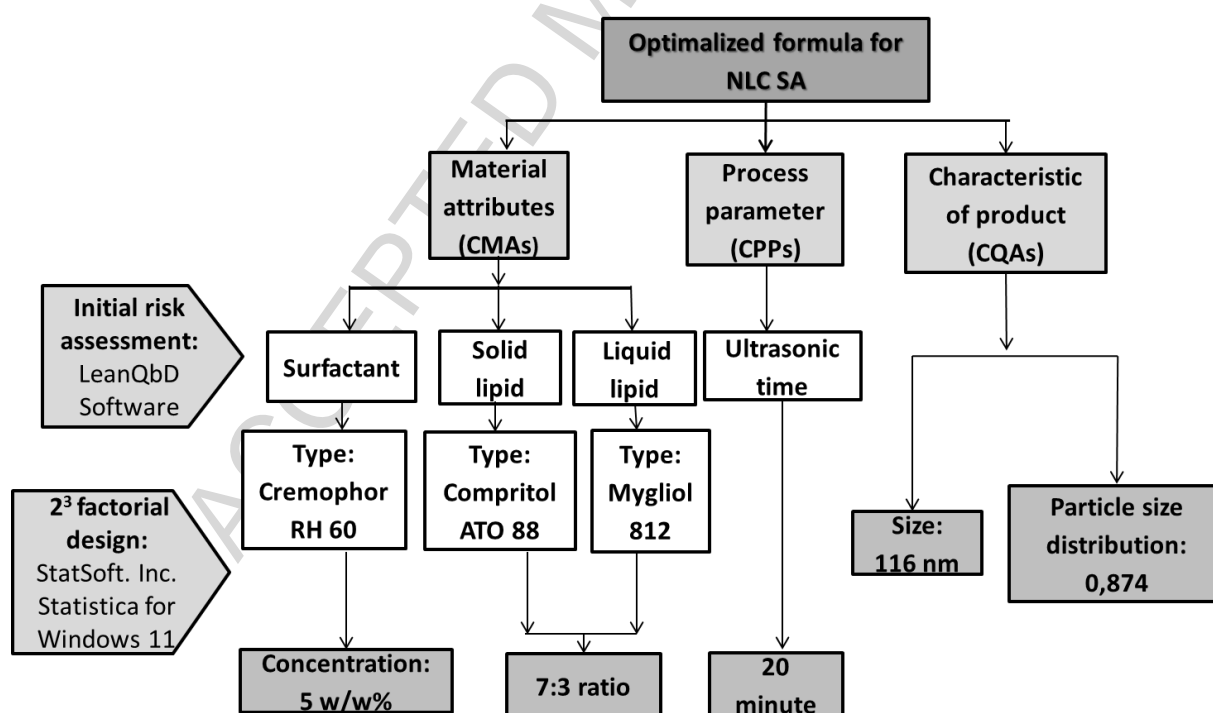
**Fig.9.** Response surface (3D) plot of the effects of variables on particle size distribution of the prepared NLC SA.

As seen in Fig. 9A, the combination of the highest surfactant concentration (5 w/w%) and the lowest lipid ratio (7:3) gives the smallest particle size distribution. Based on Fig. 9B, a higher surfactant concentration (5 w/w%) and a higher ultrasonication time (20 minutes) gives the smallest particle size distribution. Fig. 9C shows that the smallest particle size distribution is available with a higher ultrasonication time (20 min) and a higher lipid ratio (9:1). However, the desired particle size (100–200 nm) is achieved after 10 minutes at the 7:3 lipid ratio, while another 20 minutes of ultrasonication only slightly reduces particle size. Consequently, choosing the 7:3 lipid ratio is recommended. The standardized effect of the independent variables and their interaction on the dependent variable was also evaluated by preparing a Pareto chart. As illustrated by the Pareto charts in Fig. 10, only those factors crossing the vertical line have a significant effect. Accordingly, the surfactant concentration was found to have the highest and a significant effect for mean particle size and particle size distribution.



**Fig.10.** Pareto chart: (A) mean particles size and (B) particle size distribution.

According to these results, the lowest particle size can be achieved with a surfactant concentration of 5%, a lipid ratio of 7:3 and 20 min sonification time. These parameters apply for the formulation ‘NLC 8’, which was selected for further investigations. After selecting the optimal formula on the basis of the  $2^3$  factorial design using response surface methodology and Pareto charts, we validated the preparation method. The validation process included the reproduction of the optimal formulation (NLC 8), and its characteristics, including particle size and particle size distribution were analyzed. Fig. 11 shows the fault tree for the optimized formula of NLC SA.



**Fig.11.** Optimized formula for NLC SA and the parameters investigated ( $n=2$ ).

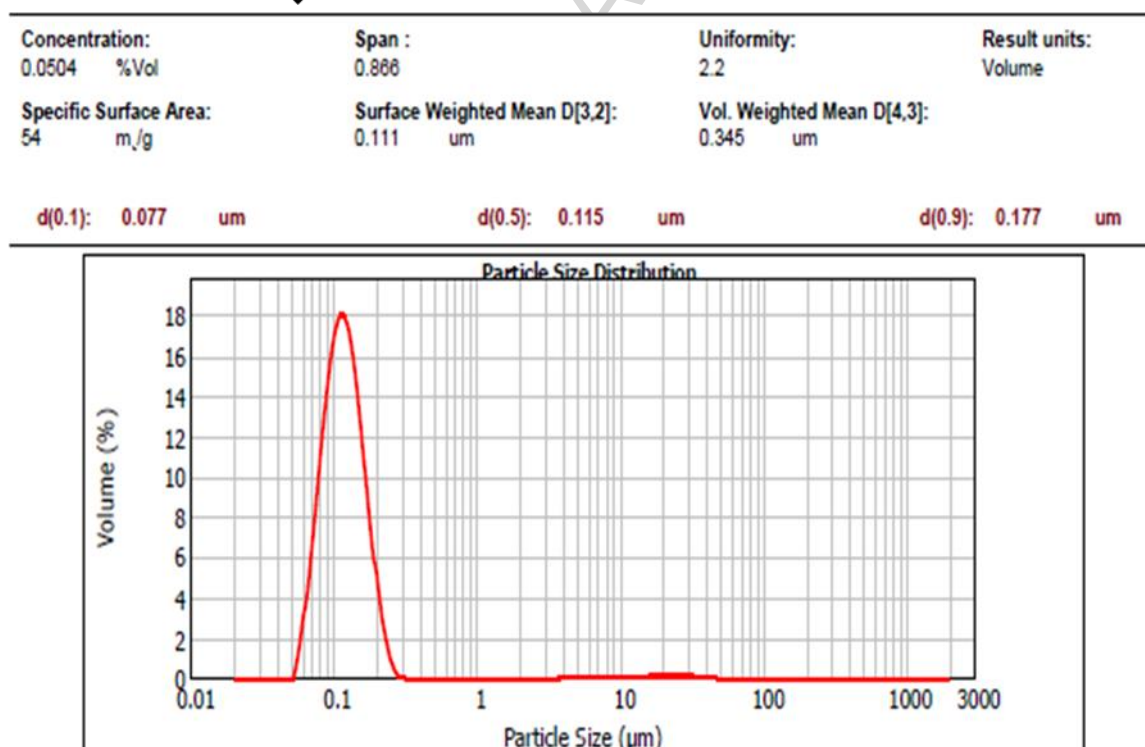


### 3.5. Characterization of the optimized NLC SA

#### 3.5.1. Particle size analysis and validation of the preparation method

The particle size analysis confirmed the experimental results for the mean particle size and the particle size distribution as shown in Fig. 11 and Fig. 12. We have also demonstrated that these mean particle size ( $d(0.5)$ ) and particle size distribution (span) values are appropriate and they meet the particle size requirements for nanosystems NLC (100–200 nm) as defined in QTPP. The experimental results (114 nm) of the validation process are comparable to the predicted values (116 nm). Thus, the preparation method defined by the risk assessment was found to be appropriate.

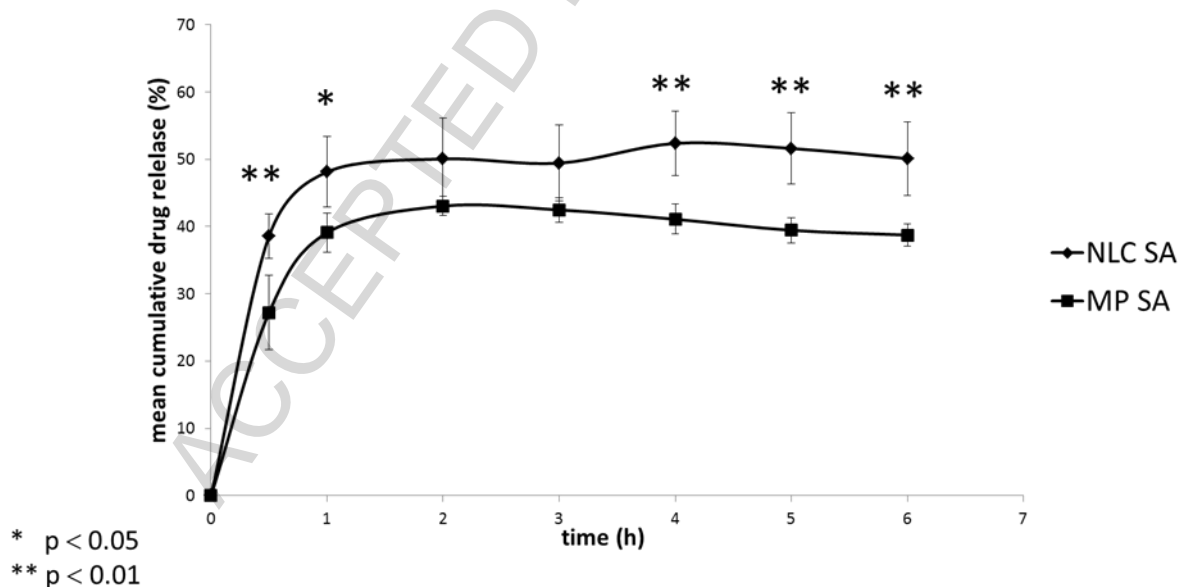
	8. NLC /1	8. NLC/2	8. NLC/3	Mean particle size	SD
d(0.1)	0.077	0.075	0.076	0.076	0.001
d(0.5)	0.115	0.111	0.116	0.114	0.002646
d(0.9)	0.177	0.168	0.177	0.174	0.005196
Span	0.866	0.840	0.865	0.857	0.014731



**Fig.12.** Results of the particle size analysis for the optimized formula of NLC SA

### 3.5.2. *In vitro* drug diffusion

*In vitro* diffusion of salicylic acid through the artificial membrane was examined using the NLC composition and the micro-particle reference preparation containing salicylic acid (MP SA). The extent of *in vitro* diffusion was calculated as the mean cumulative amount diffused at each sampling time during a period of 6 h (Fig. 13). Presently, the *in vitro* diffusion study used an “in process control” method to check the difference between the microparticle and nanoparticle formulation. As seen in Fig. 13, the amount of API released from the NLC after 6 h was significantly higher than that released from the reference preparation. Furthermore, 48% of the API incorporated into the nanostructured lipid carriers (NLC) was found to be dissolved within 60 minutes, while the drug release was only 39% in case of MP SA reference product. These results confirm that the target NLC SA was a nanosized formula with sustained drug release as defined in the QTPP, characterized by a minimum of 50% dissolution efficiency at 60 minutes. These results further confirm that particle size highly influences drug release.



**Fig.13.** Release profiles of salicylic acid from the micro-particle and the NLC system

#### 4. Conclusion

The present paper describes a successfully optimized formula of nanostructured lipid carriers containing salicylic acid, prepared by applying the QbD concept on the development process. Based on the results of the initial risk assessment, preformulation studies and experimental design (DoE), the optimal composition for the salicylic acid-loaded NLC system was defined. Initial risk assessment was carried out to select the CMA and CPP parameters in order to identify factors affecting the CQAs. Based on the results of the initial risk assessment, three CQAs, namely particle size, particle size distribution and aggregation were found to be highly critical attributes for the NLC SA. Furthermore, five CQAs, such as the dissolution rate, dissolution efficiency, pH, solubility of the API and entrapment efficiency were found to be attributes of medium influence. The initial risk assessment also revealed that three factors, namely surfactant concentration, the solid lipid/liquid lipid ratio and ultrasonication time were highly critical factors for the CQAs. In order to assure the desired NLC SA quality, the individual effects of CMAs and CPPs on CQA were evaluated by the  $2^3$  factorial design. The most critical CMAs and CPPs were chosen to be the independent variables and the CQAs were chosen to be the dependent variables in the  $2^3$  factorial design process. The optimized formula of NLC SA was found to be characterized by a surfactant concentration of 5%, a 7:3 lipid ratio and 20 min ultrasonification time. The above-mentioned risk assessment protocol (including the Ishikawa diagram, the risk estimate matrix and the Pareto chart as calculated by the LeanQbD Software), as well as the factorial design was used for each step of formula optimization. Future development of NLC SA systems would be greatly facilitated by an enhanced practical understanding of the behavior of these systems. The risk assessment method is a helpful tool for the optimal product development process, allowing to define the optimal NLC formulation. Based on these results, a promising salicylic acid-loaded NLC formulation could be developed which can function as a potential dermal drug delivery system in the treatment of acne, psoriasis and eczema.

#### References

- Araújo, J., et al., 2012. Release profile and transscleral permeation of triamcinolone acetonide loaded nanostructured lipid carriers (TA-NLC): in vitro and ex vivo studies. *Nanomedicine*, 8(6), 1034-1041. doi: 10.1016/j.nano.2011.10.015.
- Baroli, B., 2010, Penetration of nanoparticles and nanomaterials in the skin: fiction or reality? *J. of Pharm. Sci.*, 99(1), 21-50. DOI: 10.1002/jps.21817
- Beg, S. et al., 2015, QbD-based systematic development of novel optimized solid self-nanoemulsifying drug delivery systems (SNEDDS) of lovastatin with enhanced

- biopharmaceutical performance, Drug Delivery, 22(6), 765-784. doi: 10.3109/10717544.2014.900154
- Casanova F. and Santos L., 2016. Encapsulation of cosmetic active ingredients for topical application – a review. Journal of Microencapsulation. 33(1) <http://dx.doi.org/10.3109/02652048.2015.1115900>
- Cevc, G. and Vierl, U., 2010, Nanotechnology and the transdermal route: A state of the art review and critical appraisal. Journal of Controlled Release, 141(3), 277-299. doi: 10.1016/j.jconrel.2009.10.016
- EMA/CHMP, 2013, European Medicines Agency, Reflection paper on the data requirements for intravenous liposomal products developed with reference to an innovator liposomal product, Available from: [www.ema.europa.eu/docs/en\\_GB/document.../WC500156162.pdf](http://www.ema.europa.eu/docs/en_GB/document.../WC500156162.pdf) (accessed 12.07.2016)
- FDA, 2011, Food and Drug Administration, Guidance for Industry Topical Acne Drug Products for Over-the-Counter Human Use — Revision of Labeling and Classification of Benzoyl Peroxide as Safe and Effective Small Entity Compliance Guide, Available from: [www.fda.gov](http://www.fda.gov) (assessed 29.08.2016)
- ICH Q8(R2), 2009 Guidance for industry Q8(R2) Pharmaceutical development, August, Available from: <http://www.ich.org/products/guidelines/quality/article/quality-guidelines.html> (accessed 11.07.16)
- ICH Q9, 2006 Quality Risk Management Guidance for industry dated June, Available from: [www.fda.gov/downloads/Drugs/.../Guidances/ucm073511.pdf](http://www.fda.gov/downloads/Drugs/.../Guidances/ucm073511.pdf) (accessed 11.07.16)
- Kan, S. et al., 2014, A quality by design (QbD) case study on enteric-coated pellets: Screening of critical variables and establishment of design space at laboratory scale, Asian J. of Pharm. Sci. 9, 268-278. doi:10.1016/j.ajps.2014.07.005
- Kheradmandnia, S., et al., 2010. Preparation and characterization of ketoprofen-loaded solid lipid nanoparticles made from beeswax and carnauba wax. Nanomedicine, 6(6), 753-759. doi:10.1016/j.nano.2010.06.003
- Kovacs, A. and Erős, I. and Csóka, I., 2016. Optimization and development of stable w/o/w cosmetic multiple emulsions by means of the Quality by Design approach, Int. J. of Cosm. Sci., 38, 128-138. doi:10.1111/ics.12248
- Lacatusu, I., et al., 2011. The encapsulation effect of UV molecular absorbers into biocompatible lipid nanoparticles, Nanoscale research letters, 6(1), 73. DOI: 10.1186/1556-276X-6-73

- McGrath, J.A. and J. Uitto, J., 2010, Anatomy and Organization of Human Skin, in Burns, T., Breathnach, S., Cox, N., Griffiths C. (eds), *Rook's Textbook of Dermatology*, Wiley-Blackwell. pp. 1-53. DOI: 10.1002/9781444317633
- Müller, R.H. et al., 2002, Solid lipid nanoparticles (SLN) and nanostructured lipid carriers (NLC) in cosmetic and dermatological preparations, *Advanced Drug Delivery*, 54, 131-155.
- Müller, R.H. et al., 2014. Lipid nanoparticles (SLN, NLC) for innovative consumer care and household products. *H&PC Today* 9 (2), 18–24.
- Pallagi, E. et al., 2015. Adaptation of the quality by design concept in early pharmaceutical development of an intranasal nanosized formulation, *Int. J. of Pharm.* 491, 384-392. doi: 10.1016/j.ijpharm.2015.06.018.
- Pardeike, J., et al., 2009, Lipid nanoparticles (SLN, NLC) in cosmetic and pharmaceutical dermal products, *Int. J. of Pharm.*, 366, 170-184. doi: 10.1016/j.ijpharm.2008.10.003.
- Patel, J.N. et al., 2012. *Int. J. of Pharm. & Life Sci. (IJPLS)*, Vol. 3, Issue 2: Feb.: 2012, 1459-1469-1459
- Prow, T.W., et al., 2011, Nanoparticles and microparticles for skin drug delivery, *Advanced drug delivery reviews*, 63(6), 470-491.
- Shah, B. et al., 2015, Application of quality by design approach for intranasal delivery of rivastigmine loaded solid lipid nanoparticles: Effect on formulation and characterization parameters, *Eur. J. Pharm. Sci.*, 78, 54-66. <http://dx.doi.org/10.1016/j.ejps.2015.07.002>
- Singh, B. et al 2011, Developing micro-/nanoparticulate drug delivery systems using "design of experiments", *Int. J. of Pharm. Investigation*, 1(2), 75-87.; doi: 10.4103/2230-973X.82395
- Subedi, R.K. et al., 2009, Preparation and characterization of solid lipid nanoparticles loaded with doxorubicin, *Eur. J. of Pharm. Sci.*, 37, 508–513.; doi: 10.1016/j.ejps.2009.04.008
- Sütő, B. et al., 2015a, Optimisation Ibuprofen loaded nanostructured lipid carrier with 2<sup>3</sup> factorial design, *Chemical Engineering Research and Design*, 104, 488-496. <http://dx.doi.org/10.1016/j.cherd.2015.09.010>
- Sütő, B. et al., 2015b. Development of ibuprofen-loaded nanostructured lipid carrier-based gels: characterization and investigation of in vitro and in vivo penetration through the skin, *Int. J. of Nanomedicine*, 11, 1201-1212. <http://dx.doi.org/10.2147/IJN.S99198>
- Xu, X. et al., 2011, A quality by design (QbD) case study on liposomes containing hydrophilic API: I. Formulation, processing design and risk assessment, *Int. J. of Pharm.*, 419, 52– 59. <http://dx.doi.org/10.1016/j.ijpharm.2011.07.012>
- Xu, X. et al., 2012, A quality by design (QbD) case study on liposomes containing hydrophilic API: II. Screening of critical variables, and establishment of design space at

laboratory scale, Int. J. of Pharm., 423, 543-553.

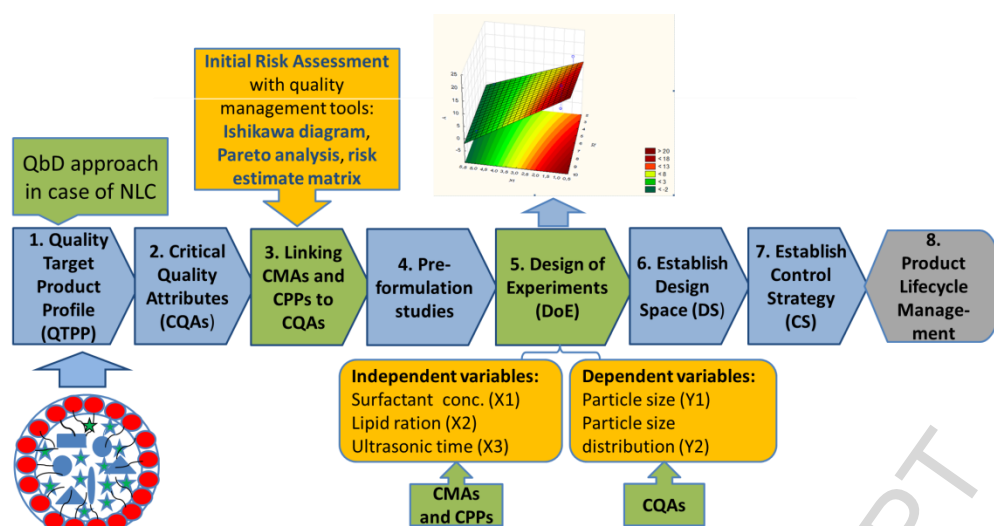
<http://dx.doi.org/10.1016/j.ijpharm.2011.11.036>

Yang, X., et al., 2013, Preparation and characterization of 4-dedimethylamino sancycline (CMT-3) loaded nanostructured lipid carrier (CMT-3/NLC) formulations. Int. J. of Pharm., 450(1-2), 225-234.

Wang, J. et al, 2015, Application of quality by design (QbD) to formulation and processing of naproxen pellets by extrusion-spheronization, Pharm. Dev. Technol., 20(2), 246-256. <http://dx.doi.org/10.3109/10837450.2014.908300>

Wissing, S.A. and Müller, R.H., 2002a, The influence of the crystallinity of lipid nanoparticles on their occlusive properties, Int. J. of Pharm., 242(1-2), 377-379. DOI: 10.1016/S0378-5173(02)00220-X

Wissing, S.A. and Müller, R.H., 2002b, Solid lipid nanoparticles as carrier for sunscreens: in vitro release and in vivo skin penetration, J. Control Release, 81(3), 225-233. DOI: 10.1016/S0168-3659(02)00056-1



Graphical abstract